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Anticancer Principles from Salivary Gland Extract of *Octopus ageina*

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Abstract: The present study was carried out to evaluate the anticancer potential of salivary gland extracts of *Octopus ageina* on *in vitro* and *in vivo* cancer models. LD_{50} values of both anterior (AGE) and posterior gland extract (PGE) were analyzed using Swiss Albino mice. Different concentration of sublethal dose was subjected to hemolytic assay using 1% human erythrocytes. Since PGE did not show any hemolysis at concentration below 400 µg, it was chosen for the anticancer studies. Preliminary anticancer effect was tested on COLO 205 cells at three different concentrations, viz., 50, 75 and 100 µg mL^{-1} medium. Among the above three doses, 100 µg mL^{-1} medium was found to inhibit cell growth effectively after 24 h. The same effective dose was used to confirm the anticancer potential of PGE against 1.2-dimethylhydrazine (DMH) induced colon cancer in male Albino Wistar rats. After 30 weeks of the experimental period PGE showed promising effects by reduced tumor incidence and mean tumor size. The β-glucuronidase (intestine and colon) and mucinase (Colon and fecal contents) activity were significantly reduced after treatment with PGE when compared to the DMH treated rats. The above anticancer effect of PGE was further confirmed by the histopathological changes of the colon. Thus, PGE proves itself to have potent anticancer substance and further studies are warranted to isolate and identify the active component.

Key words: *Octopus ageina*, COLO 205, DMH, Colon cancer, antiproliferation

Introduction

Many organisms that have prevailed through evolution are in many cases equipped with an impressive array of toxins for defense and offense (Walker *et al*., 1990). Among them, the phylum mollusca contain two notable classes with toxic species: these are gastropod and cephalopod and a variety of pharmacological actions are induced by the action of toxins found in mollusks. The cephalotoxin have more complex actions and they are often species specific (Hashimoto, 1979). The toxic principles in saliva of the octopus are rationale. Many studies have shown that cephalopod saliva generally has a powerful paralyzing and lethal effect on their crustacean prey (Songdahl and Shapiro, 1974).

The most studied cephalopod toxin is maculotoxin (tetrodotoxin) from blue-ringed Octopus, *Hapalochlaena maculosa* and puffer fish, which sometimes causes human fatalities. In addition, many active protein fractions has been identified from different cephalopods like *Sepia officinalis*, *Octopus dolfleini* and proved to be a potent toxin against crustaceans (Songdahl and Shapiro, 1974). Eledoisin,

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a hypotensive and smooth muscle stimulant was isolated from acetone extract of *Eledone moschata* and *Eledone aldrovand* (Anastasi and Espamer, 1963). α- and β- cephalotoxin with molecular weight 91.2 and 33.9 kDa were extracted from posterior gland of *O. vulgaris* (Cariello and Zanetti, 1977). In this order the toxins from the *Octopus ageina*, which has many bioactive substances is least studied.

The cephalopod, *O. aegina* is a benthic, not highly cryptic species, common on the continental shelf from 30 to 120 m depth. Its geographical distribution ranges from Western pacific, Indian ocean, Red sea, Japan to Mozambique. The maximum mantle length is 10 cm (total length 30 cm). Commercially, *O. aegina* is the commonest species in Indo Malaysian markets; it is trawled on the continental shelf or caught with traps and on hook and line (FAO, 1984).

The venom apparatus of the cephalopod comprises the anterior and posterior salivary gland and are directly associated with the buccal mass and mandible. The venom apparatus is an intimate part of the digestive function (Russell, 1984). Of the two salivary glands, posterior gland is found to possess numerous bioactive substances like, tyramine (Henze, 1913), histamine (Botazzi and Valentini, 1992), octapamine, enteramine (Espamer, 1953).

The approach of testing venoms as antitumour agents dates back to the beginning of the past century, when Calmette *et al.* (1933) reported on the antitumour activity of snake venom, (Naja species venom) on adenocarcinoma cells. Since then many reports have appeared on this subject and controversies still exist (Shiau-Lin *et al.*, 1976; Baldi *et al.*, 1988; Silva, 1995). Proteinacious venom from several animals like bee, snake, spider etc. were reported to have excellent cytotoxicity in cultured cancer cell lines and reduction of tumor growth in mice models (Orsolie *et al.*, 2003; Abu-Sinna *et al.*, 2003).

Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives every year (Hemminkin and Mutanen, 2001). An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents (alone or in combination) to block the development of cancer in humans. Colon cancer is the second most common cause of cancer death worldwide and third most in Western societies (Shike *et al.*, 1990).

In the recent past, crotoxin, an anticancer agent from *Crotalus durisssus tinifinicus* venom a cytotoxic phospholipase A_2 is under phase I clinical trial (Cura *et al.*, 2002). However, the marked curative properties of such venoms are always hindered by their high toxicities; in most cases the venoms are cytotoxic to normal cells same as they are toxic to the malignant cells. Hence a comparatively less toxic and edible species, *O. ageina* is chosen for this study and tested against colon cancer using both *in vitro* and *in vivo* models. Understanding the mode of formation and evolution of experimental colonic neoplasm may lead us to find better methods for preventing and treating colon cancer.

Materials and Methods

Materials

COLO 205 (Human colon cancer cell line) was obtained from NCCS pune. 1,2-dimethylhydrazine(DMH), p-nitrophenyl β-D-glucuronide and mucin were purchased from Sigma Chemical Company; St. Louis, MO, USA. [^{3}H]thymidine was from Amersham, UK. All other chemicals used were of analytical grade and obtained from S.D Fine Laboratories, Mumbai.

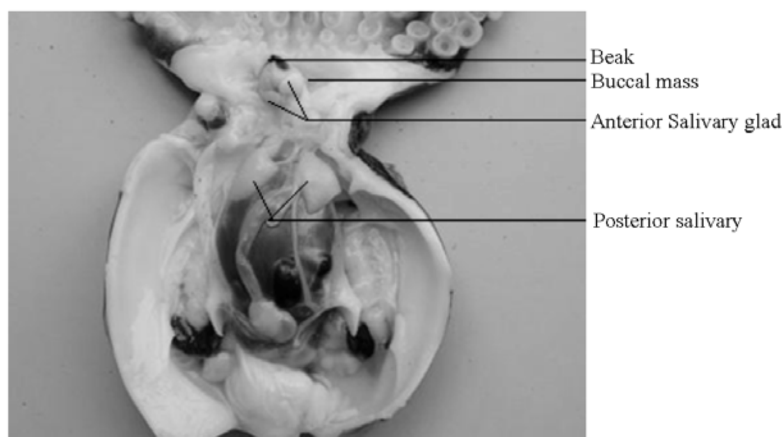

Fig. 1: Showing the anterior and posterior salivary glands of *Octopus ageina*

Preparation of Crude Extract

The *Octopus agenia* was collected from the local market and transported to the laboratory with ice. One hundred animals were dissected and the anterior and posterior salivary glands (Fig. 1) were removed separately and homogenized in Phosphate buffered saline (PBS), at pH 7.2 in 4°C. The homogenate was then centrifuged in the refrigerated centrifuge and the supernatant was collected, lyophilized and stored at -20°C till use. The protein was estimated by Lowry *et al.* (1951) and the lyophilized sample was dissolved in PBS containing 5 mg mL^{-1}.

Toxicity Assay

The LD_{50} was determined as described by Kulkarni (1999), by the method of Litchfield and Wilcox (1949). Male albino Swiss mice of (20±2 g) were used and the study was approved by the Institutional Ethical Committee.

Hemolytic Assay

Hemolytic assay was performed with the human erythrocytes by the method described by Paniprasad and Venkateshwaran (1997). In brief, the assay was performed in the V shaped 96 well microtitre plate. Serial two fold dilutions of the venom extract (1 mg of the AGE and PGE in 1 mL PBS) were made in PBS (pH 7.2) starting from 1:2. An equal volume of 1% human RBC was added to all wells. The plates were shaken gently to mix the extract and RBC kept for 2 h before reading the results. One percent RBC with distilled water served as blank and with PBS as negative control.

In Vitro Antiproliferative Assay Using [^{3}H]thymidine Incorporation

COLO 205 was maintained in RPMI 1640 supplemented with 10% Fetal calf serum, amphotericin (3 μg mL^{-1}), gentamycin (400 μg mL^{-1}), streptomycin (250 μg mL^{-1}), penicillin (250 units mL^{-1}) in a carbon dioxide incubator at 5% CO_2.

In a six well plates [^{3}H]thymidine (1 μci per 1 mL of medium) was added to the medium in which the cell line was already maintained. Twenty microliter of different concentration (25, 50, 100 and 150 μg mL^{-1}) of the PGE was added to the cells. A same volume of buffer was added to the control well. The cultures were trypsinized at the desired time points, pelleted and washed sequentially with

10 and 5% tricholoroacetic acid and solublized in 0.1% sodium hydroxide and 0.025% sodium dodecyl sulphate solution. The radioactivity of the samples was measured in the Packard, TopCount. NXT™ Liquid scintillation counter and expressed as cpm/mg protein.

In vivo Anticancer Studies

Animals and Animal Husbandry

Male adult Wistar rats of body weight 120-150 g were obtained from the Central Animal House, Rajah Muthiah Medical College, Annamalai University, Annamalai Nagar, (acclimatized with control diet for 6 days). Rats were assorted into experimental groups as given in experimental design. Animals were maintained as per the principles and guidelines of the Ethical Committee of Animal Care of Annamalai University in accordance with the Indian National Law on animal care and use. The animals were housed four per cage in polypropylene cages with a wire mesh top and a hygienic bed of husk in a specific-pathogen free animal room under controlled conditions on a 12 h light/12 h dark cycle, with a temperature of 28±1°C and a relative humidity of 50±10% till the end of the experimental period.

Carcinogen Administration

All the animals in groups II to VI received 20 mg kg^{-1} body weight DMH through subcutaneous injection once in a week for the first 15 weeks. Prior to injection, 1,2-dimethylhydrazine (DMH) was dissolved in 1 mM EDTA, the pH was adjusted to 6.5 with 1 mM sodium hydroxide and was used immediately. The animals were separated into different four groups.

Experimental Design

Group 1: Control rats, which received standard pellet diet, water ***ad libitum*** and 0.2 mL PBS (i.p) throughout the experiment.

Group 2: Rats were administrated 20 mg kg^{-1} b.w DMH (s.c) once in a week for first 15 weeks and continued the standard pellet diet.

Group 3: The animals were administered DMH (20 mg kg^{-1} b.w) for first 15 weeks along with (PGE) 100 µg kg^{-1}.b.w (i.p) till the end of the experiment.

Group 4: The animals were administered 100 µg PGE kg^{-1}.b.w (i.p) till the end of the experiment.

Tumor Analysis

At the end of the experiment (30 weeks of time) the animals were fasted overnight, anesthetized and sacrificed by cervical decapitation. The presence of tumors was carefully noted; tumor-bearing areas and areas suspected of bearing lesions were excised and embedded in paraffin. Five micrometer, thick sections were cut to expose the central part of the tumor or the stalk and were stained with hematoxylin and eosin. In addition to tumors, flat mucosa from each segment of the fixed colon with no visible tumors was cut into 3 µm wide strips, which were embedded in paraffin. Thin sections were prepared and examined microscopically.

Biochemical Analysis

Fecal and the mucosal scrapings of proximal and distal colon were used for the analysis of mucinase activity (Shiau *et al.*, 1983). Colon contents, distal and proximal colon were analysed for β-glucuronidase activity (Shiau *et al.*, 1983). Proteins were estimated by the method of Lowry *et al*, (1951).

Statistical Analysis

All the values are expressed as means±SD of 6 animals in each group. Data within the groups were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. A value of $p<0.05$ was considered statistically significant.

Results

Toxicity Studies

The lethal dose of the two salivary glands extract, anterior and posterior was found to be 3.0 and 1.5 mg protein kg^{-1} b.w, respectively. Table 1 shows the hemolytic activity of both anterior (AGE) and posterior gland extracts (PGE) on 1% human erythrocytes. PGE showed hemolytic activity only above 0.4 mg protein and below which no lysis was observed. Where as, AGE showed hemolysis starting from 0.1 mg protein and not suitable for the further study. Hence, PGE alone was used for the further analysis.

Antiproliferative Studies

The preliminary antiproliferative effect of PGE on COLO 205 is shown in Table 2. [^{3}H] thymidine incorporation studies were conducted in three time points 12, 24 and 48 h. There was marked inhibition of proliferation by PGE at 24 h. The results are expressed as counts of [^{3}H] thymidine incorporated into the actively proliferating cells per min per mg protein of the cell mass. In the Table 2, the cell control and PBS control shows the maximum counts of [^{3}H]thymidine (29000 cpm/mg protein) when compared to the treatment groups. Among the different treatment, cells treated with 100 µg mL^{-1} of PGE showed the maximum inhibition (9000 CPM/mg protein). Since this sub lethal dose (0.1% of lethal dose), did not show any hemolysis and found to have effective antiproliferative effect at *in vitro* condition, it was used for the *in vivo* study.

Tumour Incidence

Table 3 shows the incidence of colonic neoplasms in control and experimental animals. The incidence of colonic neoplasms was 100% in DMH treated rats i.e., all the animals, which received DMH has produced tumor with the average of 2 cm in size. However the incidence was reduced to 16.6% in DMH + PGE treated rats with the average size of only 0.4 cm. No tumor was noticed In group 4, control rats, which received PGE alone for the entire period of the study.

Table 1: Hemolytic activity of *Anterior* and *Posterior* salivary gland extract on human erythrocytes

Concentration of extract (mg)	Hemolysis by AGE (%)	Hemolysis by PGE (%)
0.1	10.0±0.1	ND
0.2	19.0±1.5	ND
0.3	38.5±2.9	9.0±0.8
0.4	76.9±8.0	14.0±1.2

ND-Not detected

Table 2: Antiproliferative effect of PGE on COLO- 205 cells at 24 h

Concentration of PGE µg mL^{-1}	cpm/mg protein
Cell control	2900±1890
Buffer control	29500±2000
50	20000±1900
75	13000±1250
100	9000±855

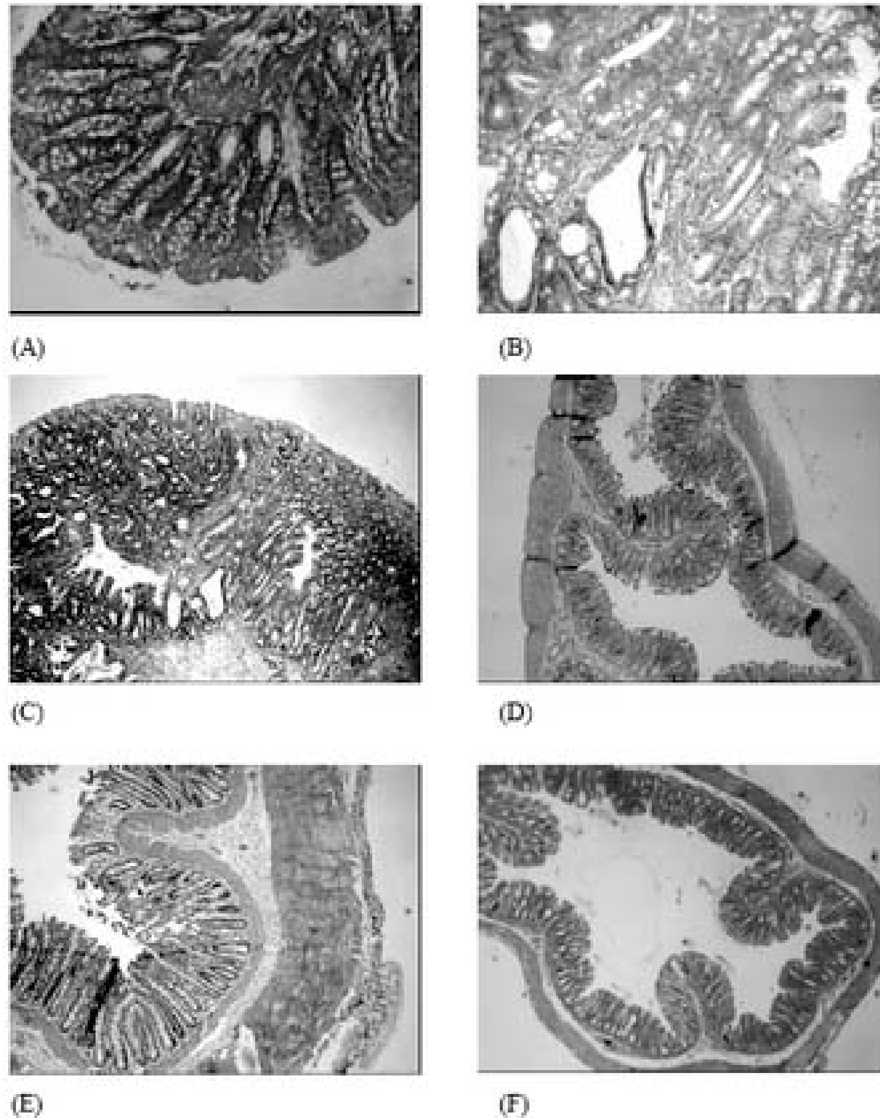

Fig. 2: Showing the histological section of colon from the normal, DMH, DMH + PGE and PGE treated rats, (A) Colon of control rats with normal mucosal and submucosal layer (40×), (B) Colon of DMH treated rats showing adenocarcinoma with papillary pattern and dysplastic zone (20×), (C) Colon of DMH treated rats showing carcinoma glands filled with mucin and lined with pleomorphic cells (40×), (D,E) Colon of DMH+PGE treated rats showing lymphocyte infiltration without any well defined tumor (10× and 20×), (F) Colon of PGE treated rats showing normal mucosal fold (20×)

Table 3: Showing the incidence of colonic neoplasms DMH and PGE treated groups

Groups	Treatments	No. of rats examined	No. of rats with colonic tumors	Tumour incidence (%)	No. of tumors (average)	Tumour size (%) (average in cm)
1	Control	6	0	0	0	-
2	DMH	6	6	100	12	2
3	DMH + PGE	6	1	16.6	1	0.4
4	Control+ PGE	6	0	0	0	0

Table 4: Changes in the activity of β-glucuronidase (mg of *p*-nitrophenol liberated/h/g protein) of DMH and PGE treated groups

Groups	Distal colon	Proximal colon	Distal intestine	Proximal intestine
Control	55.5±2.9[a]	47.25±.3.9[a]	50.66±6.2[a]	53.96±7.6[a]
DMH	110.8± 10.9	58.8±3.3	59.7±4.2	64.2±8.0
DMH+PGE	61.2±6.9[a]	50.50±5.0[a]	52.52±2.72[a]	56.18±2.8[a]
Control+PGE	57.6± 3.8[a]	48.12±3.9[a]	50.25±4.8[a]	51.20±4.9[a]

*Values are mean±SD of 6 rats in each group. Values having the superscript a differ significantly from DMH treated group at $p<0.05$ (DMRT)

Table 5: Changes in the activity of mucinase (mg of glucose liberated/min/mg/protein)

Groups	Colon contents	Fecal contents
Control	1.5±0.1[a]	3.1±0.1[a]
DMH	3.6±0.1	5.2±0.7
DMH+PGE	2.3±0.1[a]	4.1±0.3[a]
Control+PGE	1.7±0.1[a]	3.2±0.3[a]

*Values are mean±SD of 6 rats in each group. Values having the superscript a differ significantly from DMH treated group at $p<0.05$ (DMRT)

Table 6: Histological changes in the colon of DMH and PGE treated rats

Macroscopy	DMH	DMH + PGE
Mean tumor size	26.8±23.6	14.1±10.2
Nature	Sessile	Pedunculated
Margin	Well defined	Ill defined
Microscopy		
Inflammatory cell infiltration into the mucosa	Marked, densely packed inflammatory cells	Scattered infiltration
Mucosal crypt architecture a) crypt branching	Small number of irregular crypts	Regular crypts
b) crypt enlargement- transitional mucosa	Observed in large numbers	Not observed
Surface epithelium		
Crypt epithelium a) Mucin production	Diffuse glands with diluted mucin	Glands are filled with mucin
b) Epithelial cell numbers	Progressive reduction in numbers	No reduction observed
c) Cell morphology 1. Atypia with nuclear hyperchromatism	Marked	Less severe
2. Pleomorphism	Marked	Less severe
3. Stratification of nuclei	Marked pseudostratificaion	Not observed
4.Mitotic figure	Numerous	Not present
Infiltration of inflammatory cells in the submucosa	Several areas shows dense infiltration	Scattered infiltrations
Lamina Propira a) Fibrosis	Observed densely eosinophilic	Not observed
b) Vascular granulation and vascular congestion	Present	Not observed

Effect PGE on β-glucuronidase and Mucinase Activity

Table 4 shows the activity of β-glucuronidase, which is mg of *p*-nitrophenol liberated/min/h/g protein in colon and intestine of both control and experimental animals. When compared to normal, β-glucuronidase activity was significantly increased in DMH treated group. Whereas, the activity was significantly decreased in PGE treated intestine and colon when compared to the DMH group.

Table 5 shows the changes in the activity of mucinase in colon and fecal contents of both control and experimental rats. The mucinase activity (mg of glucose liberated/min/mg protein) were significantly increased in DMH treated rats and decreased in the colon and fecal contents of PGE treated rats.

Histopathology

The macroscopic, histopathological observations in colon of rats in different experimental groups is documented in Fig. 2 and described in Table 6. The normal mucosal and sub mucosal layers in the colon of group 1 rats are shown in Fig. 2A. The DMH treated group 2 rats (Fig. 2B and C) showed the presence of tumor with the histological features of adenocarcinoma. The group 3 rats (DMH+PGE) showed reduced tumor size, normal mucosa and regular crypts (Fig. 2D and 2E). Whereas the group 4 PGE (Fig. 2F) treated shows normal mucosal and submucosal layers with regular, well spaced crypts.

Discussion

In vitro cytotoxicity assays and *in vivo* animal evaluation are important in identifying active structures. Even after such critical evaluation, most of the available chemotherapeutic agents are not successful because they do not differentiate the normal and cancer cells.

In the present study, we are successful in evaluating that PGE of *O. ageina* at lower concentration did not affect the normal cells but inhibits the proliferation of cancer cells. But AGE was not suitable for the study as it showed hemolysis even at very low concentration. Similar results were observed by the Key *et al.* (2002) from saliva of *O. vulgaris.* PGE, which did not show hemolysis at a concentration below 400 μg it was used to analyze the anticancer properties. The three non-toxic doses, viz., 50, 75 and 100 $\mu g\ mL^{-1}$ were chosen for the *in vitro* antiproliferative studies.

Among the three doses tested, PGE at concentration of 100 $\mu g\ mL^{-1}$ was found to inhibit the cell proliferation effectively in 24 h. The actual mechanism by which it exerts the antiproliferative effect is still to be elucidated. Since the ^{3}[H] thymidine is measured for assessing the antiproliferative index, uptake of thymidine by the proliferating cells indicate amount of DNA synthesized by the new cells. As the thymidine count is low in the treated cells, the suggested mechanism is that PGE has some principle, which prevents the DNA synthesis in cells and hence blocks the cell cycle. A Similar mechanism was suggested by Abu-sinna *et al*. (2003) for the anticancer effect of the snake venom. On this basis, 100 $\mu g\ kg^{-1}$ b.w was chosen for testing the anticancer potential of PGE in the *in vivo* model.

The conformation of the antiproliferative effect of PGE was done *in vivo* using the 1,2-dimethylhydrazine induced animal model. In animal studies repeated exposure with the organotropic colon carcinogen, 1,2,-dimethylhydrazine (DMH) produces colon tumors in rodents exhibiting pathological features that are similar to sporadic forms of human colon cancer (Pozharisski *et al*., 1979; Druckrey, 1972). Most of the chemical carcinogens such as DMH require metabolic activation in order to exert their mutagenic and carcinogenic effects (Fialal, 1977). Hence after 30 weeks of experiment, we investigated the effect of PGE on colon of tumor bearing animals.

The protective effect of PGE on the DMH induced colon cancer is confirmed by the histopathological changes (Onose *et al*., 2003). The first obvious change in the treatment is % incidence of tumor and reduction in the size. Crypts are regular and well placed with intervals when compared to the DMH group. The infiltration of inflammatory cells in the submucosa shows the severity in the DMH group and these are very much reduced in the treatment group. Moreover fibrosis and vascular granulation and congestion were not observed in the PGE administered rats as that of DMH rats. Hence PGE at concentration of 100 μg kg^{-1} b.w proves itself to be a potent anticancer agent.

Bacterial enzymes are responsible for the hydrolysis of glucronide conjugation in the colon and subsequently enter the bowel, via the bile and then retoxified by the action of β-glucuronidase which shows that the bacterial micro flora is more active in the midst of a procarcinogen or a mutagen which accounts for the increased activity of the enzyme in DMH treated groups (Weisburger *et al*., 1970).

Colonic mucins are large, highly glycosylated, polymeric glycoproteins secreted by the goblet cells lining the crypt and the surface of the colonic mucosa. The intestinal microflora is capable of degrading mucin layer for their energy source so the break down of mucin exposes the underlying epithelial cells to the chain of carcinogen (Nalini *et al*., 2004). PGE administration during the entire period of the study to the DMH treated rats lowers the activities of β-glucuronidase and mucinase, which in turn reduces the toxic effect of DMH. The reduction in the activities of the both the enzymes proves the reduced toxicity of carcinogen in different parts of the colon.

Till date so many toxins and proteins have been reported to induce apoptosis in the cancer cells by activating the enzymes of the apoptotic cascade (Araki *et al*., 2002; Waida and Dowdy, 2003). In this context, Snyder *et al*. (2003) has suggested that, a central hypothesis of anti-cancer gene therapy holds that replacement of tumor suppressor gene functions in malignant cell will result in specific death or apoptosis of the cancer cell, while sparing the surrounding normal cells. We have reported the anticancer effect of a cephalopod for the first time in colon cancer model. The actual mechanism by which PGE exerts its effect is still to be elucidated. But considering the *in vitro* results, the suggested mechanism is that PGE has some principle, which blocks the DNA synthesis of continuously proliferating cells sparing the normal ones. So the reduced tumor growth may account for the reduced microbial and colonic enzymes of the treated rats.

Thus we propose that posterior salivary gland extract of the *Octopus ageina* has excellent anticancer principle, if their nature, structure and mechanism of action are revealed it will be better drug for site-specific chemotherapy.

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